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NOTES

Transforming Activity of DNA Fragments from Normal Human Lymphocytes Results from Spontaneous Activation of a c-Ha-*ras1* Gene

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An activated human Ha-*ras* gene was present in a secondary NIH 3T3 transformant isolated after serial transfection of originally low-molecular-weight DNA fragments from normal human cells. This gene appeared to have acquired its transforming properties by a spontaneous mutation in codon 12 by substitution of a deoxythymidine residue for a deoxyguanosine residue. DNA rearrangements in the flanking sequences of the transferred Ha-*ras* gene were not involved in the activation of the protooncogene.

The transforming *ras* genes contained in the DNAs of many tumorigenic human cells are able to induce malignant transformation in preneoplastic mouse NIH 3T3 cells upon transfection. These oncogenes differ from their biologically inactive counterparts (protooncogenes) by single nucleotide exchanges (for a review, see references 7, 16, and 37). Transforming activity has also been observed when multiple copies of a human Ha-*ras* protooncogene have become integrated into the NIH 3T3 genome (27) or when the transferred normal gene has been overexpressed due to the activity of a vicinal retroviral long terminal repeat sequence (6). High-molecular-weight DNA (>20 kilobases [kb]) from normal cells has no transforming activity in the NIH 3T3 transfection assay (reviewed in references 7 and 37). However, Cooper et al. reported that small DNA fragments (0.5 to 4.5 kb) from normal chicken and preneoplastic NIH 3T3 mouse cells induced morphological transformation and proliferation of NIH 3T3 cells in semisolid agar medium (8). These authors speculated that a protooncogene might be overexpressed due to fusion to an efficient promoter after integration of donor DNA in the genome of the recipient cells.

Previously we described the rare transformation of NIH 3T3 cells by fragments from normal human DNA (29). In this study we analyzed one of the secondary transformants, HL-S2, for the presence of known oncogenes by Southern blot hybridization of its genomic DNA. In addition, the transforming DNA sequence of this secondary transformant was isolated from a recombinant phage library and partially sequenced. We report here that in this case the transforming activity of human DNA fragments is due to a spontaneous mutation in a transferred human c-Ha-*ras1* gene rather than to fusion of the corresponding protooncogene to a strong promoter.

A Ha-*ras*-specific probe hybridized to 2.9-kb *Sac*I fragments in DNAs from secondary NIH 3T3 transformant HL-S2 and from two other transformants, indicating that these cells contained transferred human c-Ha-*ras1* genes (Fig. 1A). Ha-*ras*-specific transcripts were detected by Northern blot hybridization in poly(A)$^+$ RNAs from these transformants (Fig. 1B). Expression of protein p21 in NIH 3T3 transformants was demonstrated by immunoprecipitation of lysates prepared from cells labeled with [^{35}S]methionine, using an anti-p21 monoclonal antibody (Fig. 1C). The level of Ha-*ras* expression in transfected NIH 3T3 cells was found to be slightly elevated (about threefold at the mRNA level and about twofold at the protein level) compared with untransfected NIH 3T3 cells.

The detection of a transferred human Ha-*ras* gene in HL-S2 is consistent with the notion that genes of the *ras* family are preferentially detected in NIH 3T3 transfection assays (7, 37). Since the transfected human DNA ranged in size from 0.5 to 4.5 kb, it was very unlikely that any larger cellular oncogene, such as Ki-*ras* or N-*ras* had been transferred. For the same reason, we expected to find rearrangements in the flanking sequences of the transferred Ha-*ras* gene. The human donor DNA contained the 21-kb *Eco*RI fragment and the 6.6-kb *Bam*HI fragment when it was probed with v-Ha-*ras* DNA (data not shown).

The transforming DNA sequence was isolated from a recombinant phage library constructed by inserting partially *Mbo*I-digested HL-S2 DNA into the *Bam*HI sites of phage vector EMBL3 (12, 28). The library was screened for the presence of human *Alu* repetitive DNA by filter hybridization (3), using BLUR-8 DNA (25) as a radioactive probe. The physical maps of two positive recombinant phages with overlapping DNA insertions are shown in Fig. 2. Clone λC2-2a contained both the transforming Ha-*ras* sequence (Table 1) and human repetitive *Alu* sequences located downstream from the oncogene. New *Eco*RI restriction sites were detected on either side of the Ha-*ras* gene, whereas the *Bam*HI sites were both deleted. The 0.9-kb *Sac*I fragment possibly carrying upstream regulatory sequences (4, 23) was retained intact, as were the four exons of the Ha-*ras* gene (Fig. 2). Human repetitive *Alu* sequences located upstream from the c-Ha-*ras1* locus in the undisturbed human genome (30) were replaced, probably by mouse DNA. Recombinant phage λC2-1b contained DNA sequences located at the 3′ end of the transferred Ha-*ras* gene (Fig. 2). A molecular analysis of this DNA showed that the region of the normal

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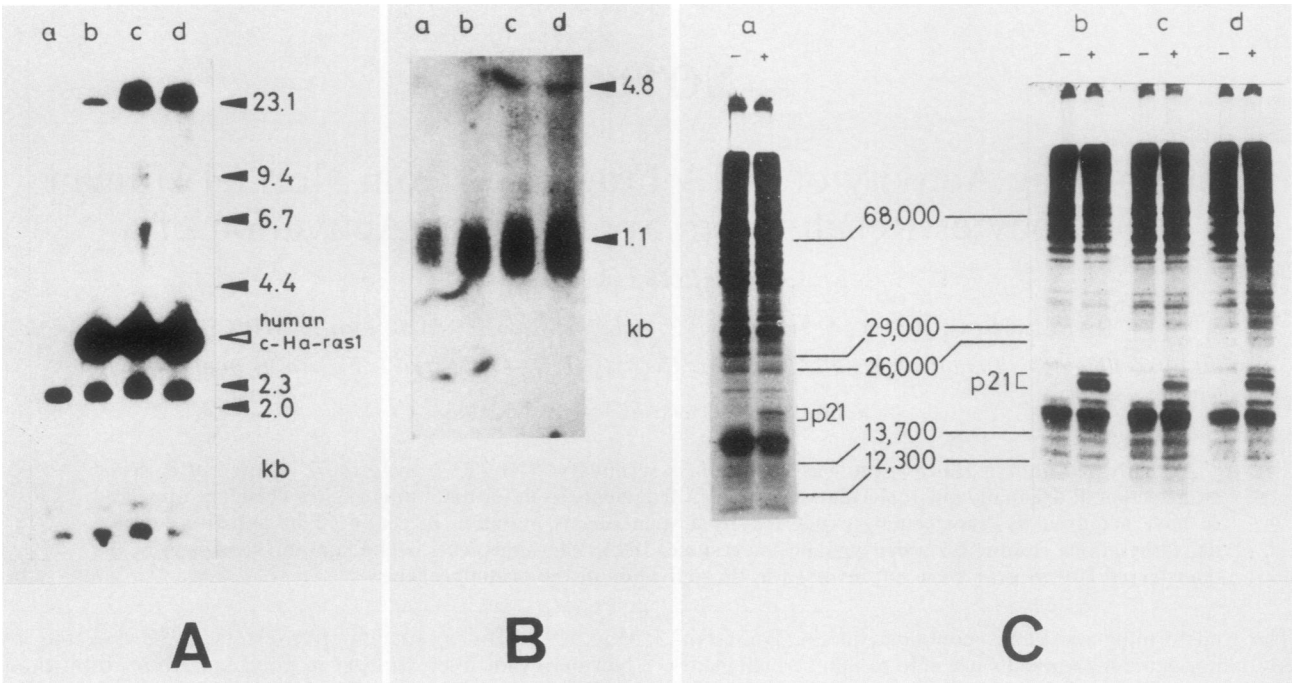

FIG. 1. Transfer and expression of human c-Ha-*ras1* DNA sequences in NIH 3T3 cells. (A) Southern blot hybridization analysis of genomic DNA containing Ha-*ras* sequences. DNA (10 μg) was digested with *Sac*I, electrophoresed through 0.8% agarose gels, and blotted onto nitrocellulose filters (35). The filters were hybridized for 36 h at 42°C with the ^{32}P-labeled nick-translated 6.6-kb *Bam*HI fragment (1×10^6 to 2×10^6 cpm/ml) containing the human Ha-*ras* gene (20) in a solution containing 50% (vol/vol) formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 1× Denhardt reagent (0.02% [wt/vol] bovine serum albumin, 0.02% [wt/vol] polyvinylpyrrolidone [molecular weight, 350,000], 0.02% [wt/vol] Ficoll [molecular weight, 400,000]), and 50 μg of sonicated salmon sperm DNA per ml. The filters were washed with 0.1× SSC containing 0.1% sodium dodecyl sulfate at 60°C. (B) Northern blot hybridization of poly(A)$^+$ RNA containing Ha-*ras* transcripts. RNA prepared as described previously (2, 9) was electrophoresed through 1% agarose–2.2 M formaldehyde gels (10) and transferred to nitrocellulose filters (35). The filters were hybridized for 36 h at 42°C with ^{32}P-labeled nick-translated v-Ha-*ras* DNA (plasmid BS9) (11) (1×10^6 to $\times 10^6$ cpm/ml) in a solution containing 50% (vol/vol) formamide, 5× SSC, 4× Denhardt reagent, 20 mM Na_2HPO_4/NaH_2PO_4 (pH 6.5), 0.1% sodium dodecyl sulfate, 100 μg of salmon sperm DNA per ml, and 10% (wt/vol) dextran sulfate. The filters were washed with 0.1× SSC containing 0.1% sodium dodecyl sulfate at 60°C. (C) Immunoprecipitation of p21 from cell lysates. Cells were labeled with 200 μCi of [^{35}S]methionine (specific activity, 1,265 Ci/mmol) in methionine-free medium for 20 h, harvested by centrifugation, and lysed as described previously (13). The lysates were immunoprecipitated with purified Y15-238 antibody (13), with purified Y15-238 antibody (+) (13), or with rat IgG (−); this was followed by addition of rabbit anti-rat immunoglobulin G and a 10% (vol/vol) suspension of formaldehyde-fixed *Staphylococcus aureus*. Washed immunoprecipitated proteins were electophoresed through 12% polyacrylamide gels (15, 31). DNA, RNA, and immunoprecipitated proteins were prepared from untransfected NIH 3T3 cells (lanes a) and from secondary NIH 3T3 transformants HL-S2 (lanes b), HL-S3 (lanes c), and HL-S8 (lanes d).

Ha-*ras* gene consisting of variable tandem repeats of a 28-base pair consensus sequence (4) was replaced by other human DNA.

A 177-base pair *Pvu*II fragment (containing 60% of exon 1) and a 446-base pair *Kpn*I-*Nco*I fragment (exon 2) were excised from a plasmid subclone of λC2-2a and cloned into phage M13mp8 (18). The complete nucleotide sequences of the insertions were determined by the dideoxy chain termination method (26). We found that the second base of codon 12 in exon 1 was thymine, thereby changing a glycine to a valine in the predicted amino acid sequence of p21. Codon 61 was not mutated in the transferred human Ha-*ras* gene (sequencing data not shown).

The genomic donor DNA from human lymphocytes used in this study for transfection of NIH 3T3 cells consisted of fragments ranging from 0.5 to 4.5 kb in size (29). Despite this fragmentation, a sufficient number of intact Ha-*ras* molecules must have been retained in the transfected DNA to give rise to a 4.5-kb *Xho*I-*Sph*I Ha-*ras* fragment that was devoid of gross structural abnormalities and was isolated from secondary transformant HL-S2. The acquisition of the transforming capacity of the transferred gene is fully explained by the single nucleotide exchange observed at position 35 of the coding sequence. Transversion mutations in this codon within the first exon of *ras* genes, with a substitution of deoxythymidine for deoxyguanosine, have been detected in human tumor cells (5, 24, 32–34). Similarly, Santos et al. (27) previously observed "spontaneous" activation of a c-Ha-*ras1* gene in a secondary NIH 3T3 transformant obtained by transfection of DNA from a primary NIH 3T3 focus containing multiple copies of the human protooncogene. This primary transfectant was isolated after transfection of a molecularly cloned human Ha-*ras* protooncogene.

We could not find any evidence for the proposition of Cooper et al. (8) that fusion of a protooncogene in the donor DNA and a regulatory sequence in the DNA of the recipient NIH 3T3 cells resulted in the acquisition of transforming properties under the experimental conditions used. Similarly, we found no evidence for activation of an endogenous mouse protooncogene by transfer and integration of a regulatory donor DNA sequence as suggested by Cooper et al.

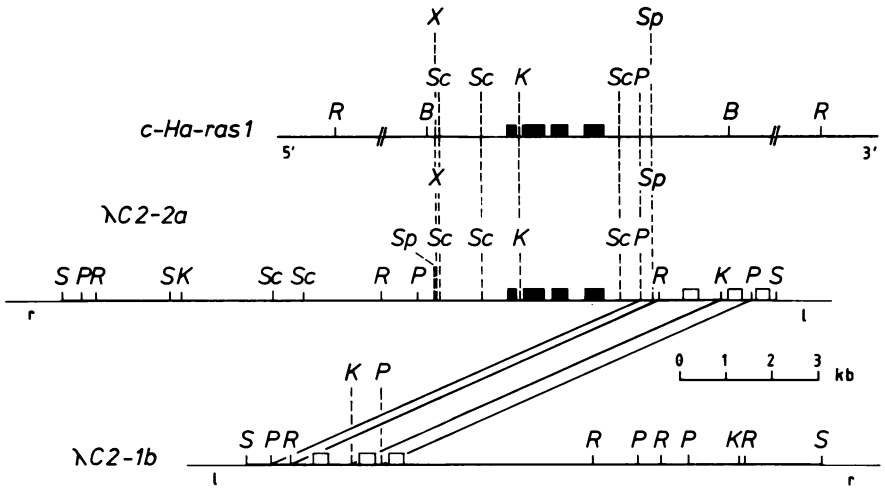

FIG. 2. Restriction endonuclease cleavage map of the human transforming Ha-*ras* sequence from HL-S2 cells. For comparison, a simplified map of the normal human c-Ha-*ras1* locus is shown. Phage arms are indicated by thin lines (r, right arm; l, left arm), and insertions of λC2-2a (15.6 kb) and λC2-1b (12.5 kb) are indicated by thick lines. The four exons of the c-Ha-*ras1* gene are indicated by solid boxes; the locations of the human repetitive *Alu* sequences are indicated by open boxes. The positions of the *Alu* sequences were not precisely mapped within the restriction fragments indicated. (*Xho*I and *Sph*I cleavage sites outside the 5.8-kb *Eco*RI fragment [Ha-*ras*], as well as *Pvu*II cleavage sites inside the 2.9-kb *Sac*I Ha-*ras* fragment, are not shown.) R, *Eco*RI; K, *Kpn*I; P, *Pvu*II; S, *Sal*I; Sc, *Sac*I; Sp, *Sph*I; X, *Xho*I; B, *Bam*HI.

(8). Rather, during or after transfection, a DNA modification affecting one of the mutational "hot spots" of the Ha-*ras* gene occurred. Selection of such a mutation could explain the previously reported rare transformation events after transfection of NIH 3T3 cells by DNA from normal cells (8, 29). It has recently been reported by workers from several laboratories that DNA transfected into mammalian cells is subject to high mutation frequencies (1, 17, 19, 22). Interestingly, the bulk of mutations involved either G · C-to-T · A transversions or G · C-to-A · T transitions, probably preceded by deletions in the transfected DNA molecules. The frequency of deletions is apparently stimulated by the introduction of double-stranded breaks and single-stranded gaps in the transfected DNA molecules (19). Therefore, it is tempting to speculate that the fragmentation by sonication of human lymphocyte DNA induces breaks in the pool of transfected Ha-*ras* sequences to an extent that transforming molecules are generated at a low frequency (29).

TABLE 1. Transforming activities of DNAs prepared from recombinant phage and plasmid subclones[a]

Donor DNA	No. of positive cultures/no. of transfected cultures	No. of foci per flask
λC2-1b	0/3[b]	0
λC2-2a	3/3[b]	>100
pC2-2a-1 5.8-kb *Eco*RI insertion[c]	2/2[d]	>100
pC2-2a-1 5.1-kb *Sph*I insertion	2/2[d]	>100
pC2-2a-1 4.5-kb *Xho*I-*Sph*I insertion	2/2[d]	>100
NIH 3T3 (control)	0/5[b]	0
208F (control)	0/5[d]	0

[a] Recipient cells were maintained in Dulbecco modified Eagle medium supplemented with 5% fetal calf serum, 5% newborn calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Transfection assays were performed as described previously (14,36). To each flask (2.5×10^5 cells) we added a calcium phosphate precipitate containing 10 μg of NIH 3T3 or 208F carrier DNA and either 300 ng of recombinant phage DNA or 100 ng of purified plasmid insertions. Foci of transformed cells were scored after 12 to 14 days.

[b] NIH 3T3 cells were used as recipients.

[c] Plasmid pC2-2a-1 was derived from λC2-2a DNA by excision of the Ha-*ras*-containing 5.8-kb *Eco*RI fragment (see Fig. 2) and subcloning into pBR328.

[d] 208F rat cells (21) were used as recipients.

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